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Constitutive expression of human intercellular adhesion molecule-1 (ICAM-1) is regulated by differentially active enhancing and silencing elements

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While expression of intracellular adhesion molecule 1 (ICAM-1; CD54) is associated with chronic inflammation and autoimmune disease, and is also found on some tumours arising from ICAM-1-negative tissues, in apparently normal tissues it is restricted to a few cell types. Levels of constitutive ICAM-1 expression correlate with the levels of ICAM-1 mRNA. In order to identify regions of the gene regulating its constitutive expression, 5.8 kb of the 5′ upstream region was studied in 16 human cell lines using transient transfection of reporter-gene constructs. Three enhancing and one silencing region were observed. While the enhancer upstream of position −1352 was active in all cells investigated, the inhibitory influence of a silencer region between positions −339 and −290 was observed only in 50% of the cells. All cells expressing low levels of ICAM-1, such as may occur in many tissues *in vivo*, lacked an active silencer. In contrast to the ICAM-1 low-expressing cells, cell lines with high constitutive ICAM-1 levels, as well as those with no ICAM-1 expression, showed an active silencer. Thus, ICAM-1 constitutive expression seems to be regulated in two different ways. The fact that silencer and enhancer activities were observed in both strongly positive and negative ICAM-1 cells, suggests that constitutive ICAM-1 expression is regulated by a balance of enhancing and silencing transcription factors and possible additional elements.

Keywords. Promoter region; regulatory sequences; transfection; luciferase plasmids; cell-adhesion molecule.

The glycoprotein intercellular adhesion molecule 1 (ICAM-1, CD54) is a member of the immunoglobulin supergene family [1, 2] and a cell-adhesion molecule that serves as a counter-receptor for a number of molecules including the leukocyte *β2* integrins LFA-1 (CD11a) and MAC-1 (CD11b) and the T-cell leukosialin CD43 [3–9]. The interaction between LFA-1 and ICAM-1 is an important step in the development of immune responses, mediating the migration of lymphocytes into the tissues, playing a costimmulatory role in T-cell activation and strengthening interactions between effector cells and their targets [10–12]. While upregulation of ICAM-1 can be induced in essentially any cell type by exposure to cytokines, in normal tissues detectable constitutive ICAM-1 expression is restricted to lymphoid follicle germinal centres, some vascular endothelia and to occasional cells in thymus, tonsils and proximal tubules in the kidney [3, 13–16]. The association of high levels of endothelial and epithelial ICAM-1 expression with a wide range of autoimmune diseases suggests that ICAM-1 plays an important role in the initiation and maintenance of these diseases [17–20]. The expression of ICAM-1 on solid tumours derived from ICAM-1-negative tissues, appears to be largely independent of the degree of leukocyte infiltration and to be rather a question of the tumour type. Thus, ICAM-1 expression is frequent on malignant melanomas and renal cell carcinomas, is found only on a minority of gastric carcinomas and breast carcinomas and very rarely on colorectal carcinomas [21–24]. While the role of ICAM-1 expressed on tumours remains unclear, it has been shown in several cases to have a prognostic significance [21, 24]. Clearly, a better understanding of how constitutive ICAM-1 expression is regulated is important in the context of both autoimmunity and host-tumour interactions. To understand how constitutive ICAM-1 expression is controlled, reporter-gene constructs of the 5′ regulatory region of the ICAM-1 gene have been transfected into various cell lines [28–32]. On the basis of studies on two cell lines, a silencing region that is active in ICAM-1-negative-cells, but inactive in ICAM-1-expressing cells has been reported [30]. In the study presented here, the expression of ICAM-1 promoter-region constructs have been characterised in 16 human cell lines deriving from different tissues. Although this silencer activity was found in 50% of the cells, it did not correlate with either the ICAM-1 phenotype or the cell type. Furthermore, a constitutively enhancing region between positions −2400 and −1352 that could override the silencer, and two additional positively acting sites downstream of position −277 were detected. A comparison of the activity of the 5′ region in ICAM-1-positive and ICAM-1-negative cell lines suggests that two distinct patterns of constitutive ICAM-1 gene regulation exist; one that is active in cells expressing low levels of ICAM-1 and one that is active in cells that are either negative for ICAM-1 or express high levels of ICAM-1.

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Abbreviations. ICAM-1, intercellular adhesion molecule-1; RLU, relative light units; MFI, mean fluorescence intensity.

MATERIALS AND METHODS

Cell culture. Human cell lines were established in our laboratory or obtained from the American Type Culture Collection,

and were cultured in RPMI 1640 medium (Biochrom) containing 5% fetal calf serum, 1 mM sodium pyruvate, 2 mM l-glutamine, 100 U/ml streptomycin and 100 U/ml penicillin.

Immunofluorescence and FACScan analysis. The first antibody used was either P3.58BA-14 anti-ICAM-1 [33] or upc10 myeloma protein (Sigma) as isotype control. The second anti-(mouse Ig) serum was fluoroisothiocyanate labelled (Dakopatts F261). The cells were examined under a fluorescence microscope and flow cytometry was performed using a FACScan (Becton Dickinson).

Northern-blot analysis. Total RNA was prepared using a modification of the caesium trifluoracetate method (Pharmacia). 20 μg RNA/lane were denatured in formaldehyde, separated on a 1.2% agarose/formaldehyde gel [34], and transferred to a Hybond-N membrane (Amersham). The 690-bp ICAM-1 cDNA *Pst*I fragment, labelled with [α-^{32}P]dATP (Amersham) using random priming [35], was used as a probe. Hybridisation and washing were carried out as described [21]. Hybridisation with an oligonucleotide (5′ CCCTGGTGAC CAGGCGGCCA ATACGGCCAA TCCGTTGACT CCGACTTTCC AC 3′) from the first exon of the glyceraldehyde-3-phosphate dehydrogenase gene [36] was used as an internal standard. The oligonucleotide was end-labelled with [α-^{32}P]dATP (Amersham) using terminal dideoxynucleotidyl transferase (Gibco-BRL) and the membrane was hybridised at 68°C in 5×NaCl/Cit (1×consists of 150 mM sodium citrate, 15 mM NaCl, pH 7.0)/10×Denhardt's solution/7% SDS/10 mM sodium phosphate, pH 7.0/10% dextran sulphate/100 μg/ml salmon sperm DNA. The membrane was washed first in 3×NaCl/Cit/10×Denhardt's solution/5% SDS/10 mM sodium phosphate, pH 7.0, at the hybridisation temperature, then in 1×NaCl/Cit/1% SDS at room temperature.

Construction and purification of luciferase reporter-gene plasmids. All constructs except pIC-5800, pIC-2400 and pIC-277ΔTATA1, have been described previously (Fig. 1B) [28]. pIC-5800 was constructed by digestion of λ clone PBL 11 (Fig. 1A; in [37] Table 1 oligonucleotides B and C were mistakenly shown as reactive) with *Sal*I, isolation of the 5.8-kb fragment and ligation with promoterless pXP-2 (kindly provided by S. K. Nordeen, Denver, USA) [38], also cut with *Sal*I. The 2.4-kb fragment was excised with *Sal*I from λ clone HWB 3R1 (Fig. 1A) and inserted into pXP-2. The fact that the clone was full length and in the correct orientation was confirmed by restriction enzyme digestion and sequencing of the cloning sites. pIC-277ΔTATA1 was constructed by deleting the *Nhe*I−*Sst*I fragment of pIC-277, then sequenced [28]. Plasmid DNA was purified by separation over two caesium chloride gradients [39]. To exclude transfection differences due to variability in DNA purification, at least two different batches were used in these studies.

Transient transfection and detection of luciferase activity of reporter-gene constructs. For transfection of the reporter-gene constructs, the Transferrinfection system (Serva) was used. 250000 cells were grown for 18 h in six-well plates (11 cm², Falcon/Becton Dickinson). The supercoiled plasmid DNA (equimolar amounts of each plasmid made up to 12 μg total DNA with pXP-2) diluted in HBS (20 mM Hepes, 150 mM sodium chloride, pH 7.3) was complexed by the poly-(L)−lysine-ferrous conjugate (16 μg/12 μg total DNA) for 30 min at room temperature, in a total volume of 0.5 ml. This was added to the cells, together with 2 ml HBS medium containing 125 μM chloroquine. Following 6 h exposure, the cells were rinsed once with warmed RPMI 1640 medium and incubated for a further 24 h in fresh RPMI 1640 medium. Cells were washed once with NaCl/P_i (15 mM potassium dihydrogen phosphate, 80 mM disodium hydrogen phosphate, 139 mM NaCl, pH 7.2) and lysed with 125 μl lysis buffer of the luciferase assay system (Promega). 20 μl cell lysate was measured for 10 s in a luminometer (LB9501, Bertold Lumat) injecting 100 μl luciferase assay reagent.

To standardise the measured relative light units (RLU), the protein content was determined according to the method of Bradford [40] using the Biorad protein assay reagent (Biorad). The standardised absolute luciferase activity was calculated by dividing the observed RLUs in 20 μl cell lysate by the protein content. Previous experiments had shown that correcting for the protein content gave rise to the same expression patterns as standardisation with the cotransfected plasmid CDM8βGal (kindly provided by A. Leutz, Berlin, Germany) and detection of β-galactosidase activity. To avoid possible titration effects when using two promoter-containing plasmids, the protein standardisation approach was routinely used. Each transfection was carried out in triplicate and at least three independent experiments were performed.

Transfection of genomic clone HWB 3R1. Transfection using lipofectin (Gibco-BRL) was performed as described [37], but using 5 μg λ DNA. ICAM-1 expression was determined by immunofluorescence and immunoperoxidase staining. The highest ICAM-1 expression was observed on day 3 after transfection.

Computer analysis. All published ICAM-1 sequences [29, 31, 32, 37] upstream of exon 1 were combined into one sequence extending from positions −1816 to +41 (the downstream transcription start site was set +1) [31]. Searches for consensus sequences in this ICAM-1 sequence were performed on a VAX/VMS machine with the GCG program package. Comparison of the transcription factors database (TFD, release 7.3) with the ICAM-1 sequence was performed on a Macintosh PC with the Macmolly program package.

RESULTS

Classification of human cell lines by their ICAM-1 expression. ICAM-1 cell surface expression was evaluated using flow cytometry on 16 cell lines of diverse histological origin (Table 1). The cells could be divided into three groups on the basis of their Δ mean fluorescence intensity (ΔMFI), i.e. MFI of anti-(ICAM-1) IgG binding minus MFI of isotype control binding: ICAM-1-negative cell lines (ΔMFI < 30), ICAM-1-positive cell lines (ΔMFI 30−200) and strongly ICAM-1-positive (ΔMFI > 200). The fluorescence intensities of the negative control upc10 (dotted line) and the anti ICAM-1 IgG (P3.58BA-14, solid line) are shown in Fig. 2 as examples of each of the three groups.

Northern-blot analysis confirmed that the cell surface levels of ICAM-1 reflect differences in steady-state mRNA levels and suggest that ICAM-1 expression is primarily regulated at the transcriptional level. As shown in Fig. 2C, cell lines 293, Colo320DM and H4 that show no ICAM-1 cell surface expression, express no detectable ICAM-1 mRNA. However, strongly positive cells Mel JuSo, A-375 and WM266-4 have high levels of mRNA, and the weakly positive cell line KATO expresses lower mRNA levels.

Reporter-gene constructs reveal silencing and enhancing elements differentially active in cells with distinct ICAM-1 expression levels. To examine whether differences in the activity of *trans*-acting proteins binding to the 5′-region DNA are responsible for differences in ICAM-1 expression, reporter-gene constructs containing up to 5.8 kb of the 5′ region of the human ICAM-1 gene were tested by transient transfection for expression in the various cell lines. Each transfection was performed in triplicate in at least three independent experiments (with the

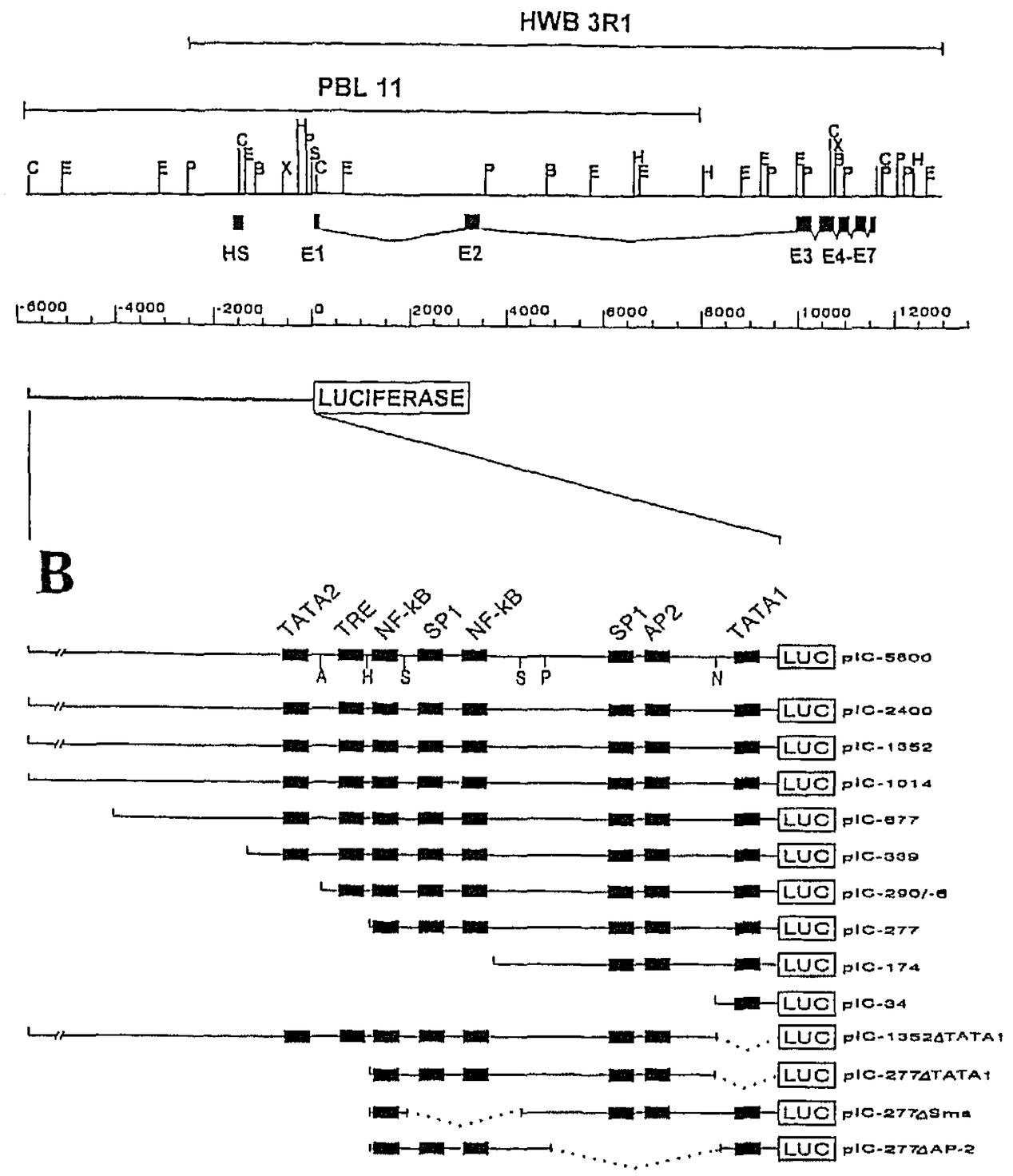

Fig. 1. Genomic structure of human ICAM-1 and reporter-gene constructs. (A) Genomic structure of the ICAM-1 gene with exons 1−7 (E1−E7). Genomic λ clone HWB 3R1 contains all exons and portions of the 5′ untranslated region as well as of the 3′ untranslated region, while PBL 11 reaches from position −5800 (the G of the downstream TGA transcription start site was set +1) to approximately position +8000 in intron 2. HS, DNAse-I hypersensitive site at position −1550. C, *Sac*I; E, *Eco*RI; P, *Pst*I; B, *Bgl*II; X, *Xho*I; H, *Hind*III; S, *Sal*I. (B) The region from position +1 to −5800 was cloned into luciferase reporter-gene construct pXP-2 (see Materials and Methods). Dotted lines, internal deletions. The TATA boxes are indicated as TATA1 (downstream) and TATA2 (upstream), while the other black boxes show potential transcription-factor-binding sites. TRE, 12-*O*-tetradecanoylphorbol-13-acetate-responsive element, SP1, Sp1; AP2, AP-2; NF-kB, NF-κB. A, *Acc*I; H, *Hind*III; S, *Sma*I; P, *Pst*I; N, *Nco*I.

exception of simian-virus-40-transformed fibroblast cell line HF/SV40, which was transfected only twice), using different DNA preparations. The measured luciferase activity (absolute expression, RLU/μg protein) of plasmid pIC-174, containing the 174-bp ICAM-1 promoter region in front of the luciferase reporter-gene, is shown in Table 1. The absolute luciferase activity measured with pIC-174 differed greatly dependent on the cell line, however independent of the ICAM-1 expression. The luciferase-containing promoterless cloning vector, pXP-2 [38], was used as a negative control. Its activity in all cell lines was below 5 RLU/μg protein and was not subtracted from test values.

The luciferase expression of a set of deletion constructs of the ICAM-1 5′ region was examined in each of the cells in Table 1. The luciferase activity of pIC-174 was set at 100% relative expression, and compared to the other constructs, shown in Fig. 2A and B for representative cell lines. In all cells examined, cells strongest luciferase expression was observed with construct pIC-5800 containing the longest 5′ region of the ICAM-1 gene. Its activity was 1.2−2.5-fold higher than luciferase expression by pIC-174. Deletion of the region upstream of position −1352 resulted in decreased reporter-gene activity in all cells (only 0.5−0.125 of the activity of pIC-5800), suggesting that this region has an enhancing influence on ICAM-1 expression. Further deletion divided the investigated cell lines into two groups. The level of expression driven by the constructs spanning the region between positions −1352 and −339 was nearly constant in both groups but on different levels. In group-A cells (Fig. 2A), plasmids containing DNA upstream of position −174 and downstream of position −1352 generated luciferase expression that was reduced by at least 50% as compared to that of the pIC-174 plasmid. Type-B cells, in contrast (Fig. 2B), revealed nearly the same luciferase expression with plasmids spanning the entire region between positions −1352 and +1. These results indicate the presence of an inhibitory element between positions −339 and −174 that is active only in type-A cells. All cells in Table 1 were found to fall either into group A or group B (partially data not shown) and, comparing this characteristic with their ICAM-1 cell surface expression, revealed an unexpected correlation. All type-B cells (WM115, IgR37, KATO, BT20, SW403, LS180, HepG2 and HF/SV40) expressed low levels of ICAM-1. However, type-A cells, in which the inhibitory effect of the region between positions −339 and −174 could be seen (Mel JuSo, WM266-4, A-375, HMCB, 293, H4, Colo320DM and

Table 1. ICAM-1 cell surface expression and luciferase activity of construct pIC-174 in the examined human cell lines. ⊿MFI = difference MFI between ICAM-1-specific antibody and isotype control. ⊿MFI > 30 was considered as positive. *n* = number of independent experiments. RLU/μg protein = RLU/μg total protein.

Cell line	Tissue	⊿MFI	*n*	pIC-174 expression	*n*
				RLU/μg protein	
Mel JuSo	melanoma	284 ± 33	11	2308 ± 699	8
WM266-4	melanoma	295 ± 31	9	2880 ± 168	5
A-375	melanoma	285 ± 68	4	212 ± 54	3
WM115	melanoma	143 ± 35	3	15 ± 3	3
KATO	stomach	156 ± 30	5	22 ± 9	4
HF/SV40	fibroblast	157 ± 49	2	17 ± 2	2
BT20	breast	141 ± 52	3	59 ± 7	4
HepG2	liver	121 ± 52	3	549 ± 124	3
LS180	colon	67 ± 6	3	44 ± 5	3
IgR37	melanoma	39 ± 13	3	101 ± 29	3
SW403	colon	39 ± 2	3	14 ± 2	3
293	fetal kidney	10 ± 20	8	2492 ± 663	8
H4	neuroglioma	9 ± 14	5	99 ± 11	5
HMCB	melanoma	5 ± 12	6	67 ± 26	3
Colo320DM	colon	5 ± 16	5	2164 ± 568	5
LoVo	colon	−8 ± 7	5	2878 ± 523	5

LoVo), were either strongly ICAM-1-positive or ICAM-1 negative.

Location of one silencing and three enhancing regions. By transfecting additional ICAM-1 5′-upstream-region constructs into the melanoma cell line Mel JuSo, the regulatory elements were localised. Mel JuSo is a strongly ICAM-1-positive type-A cell line (Table 1), that shows the inhibitory influence of the region between positions −339 and −174 (Fig. 3). High luciferase expression was obtained with plasmids pIC-5800, containing 5.8 kb of the ICAM-1 5′ upstream region, and pIC-2400 containing 2.4 kb of the ICAM-1 5′ upstream region (Fig. 3). Deletion of the region upstream of position −1352 reduced activity to approximately 10% of that of pIC-2400, indicating an enhancer between positions −2400 and −1352 that is able to override the silencer downstream.

While plasmids containing the ICAM-1 upstream region from positions −1352 to −339 showed an identical low level of reporter-gene activity, deletion of the region between positions −339 and −174 caused a fourfold increase of reporter-gene expression, similar to the expression patterns shown in Fig. 2A. This region was more closely examined using the constructs pIC-290/-6 and pIC-277 (Fig. 1B). Deletion of the region from nucleodites −339 to −290, that includes the upstream TATA box [29], caused a sevenfold increase in reporter-gene activity (Fig. 3), suggesting that the deleted region contains a binding site for the inhibitory element active in Mel JuSo. Deletion of a further 13 bp from positions −290 to −277 containing a 12-*O*-tetradecanoylphorbol-13-acetate-responsive element TRE [28], caused no significant change in luciferase expression. It therefore appears that the inhibition of the reporter-gene expression mediated by the region between positions −339 and −174 is due to a silencer-binding site located between positions −339 and −290. The sequence between positions −321 and −302 revealed homology to several known silencer-binding motifs (Table 2, see Discussion).

Deletion of the next 100 bp to position −174 still left an active promoter, but with a nearly twofold decreased activity in comparison to that of pIC-277. An internal deletion of positions −227 to −136 in pIC-277⊿Sma had nearly no effect on the luciferase expression in comparison to that of pIC-277 (Fig. 3), suggesting that the region ranging from positions −277 to −227 may be responsible for the enhanced expression of pIC-277⊿Sma and pIC-277 relative to that of pIC-174. This site contains a putative AP-2 site at position −271 (GCCTGGCC) and a potential NF-κB site at position −239 [41].

Full activity of the core promoter depended on the presence of the downstream TATA box (at −30 TATAAA) and the region between positions −108 and −34, containing putative Sp1 (−59 CCGCCC) and AP-2 (-48 CCCCCAGG) sites [41]. While pIC-277 showed full promoter activity, deletion of the region between positions −108 to −34 (pIC-277⊿AP2) led to a nearly complete loss (more than 90%) of promoter activity with only slightly higher levels of luciferase activity than plasmid pIC-34, that contains only the downstream TATA box (Fig. 3).

Deletion of the downstream TATA box from pIC-277 (construct pIC-277⊿TATA1) reduced reporter-gene activity to background levels. However, promoter activity could be restored by the addition of sequences further upstream (plasmid pIC-1352⊿TATA1), suggesting that the upstream TATA box at position −313 can also function as a transcription-initiation site (Fig. 3).

The full-length clone mediates ICAM-1 expression in ICAM-1-negative cell lines. HWB 3R1 is a full-length λ clone, containing 15.5 kb of the human ICAM-1 gene including all seven exons, six introns, a 2.4-kb upstream region and a 1.5-kb 3′ untranslated region (Fig. 1A) [37]. This clone was transfected into several ICAM-1-negative cell lines including H4, Colo320DM, 293 and HMCB. In all cell lines, ICAM-1-expressing cells were found both by immunofluorescence and by immunoperoxidase staining (data not shown). No positive cells were observed following transfection of λ DNA alone. Clone HWB 3R1, therefore, contains all information necessary for functional ICAM-1 expression, however does not reflect the *in vivo* regulation.

DISCUSSION

While human ICAM-1 expression can be induced on a variety of cell types in apparently normal tissues, it is restricted to

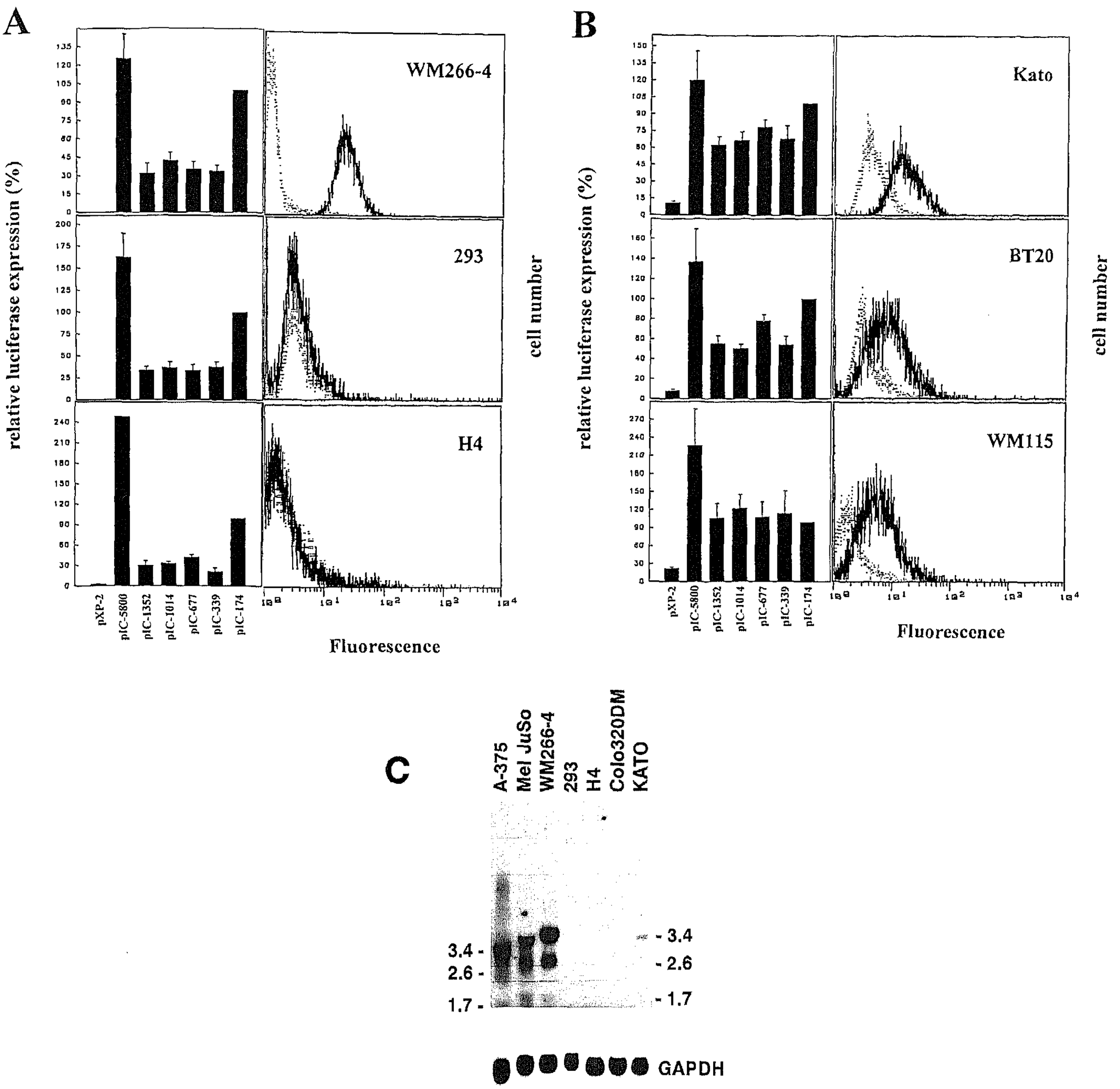

Fig. 2. Correlation between the expression pattern of the ICAM-1/luciferase constructs and the ICAM-1 cell surface expression. The right panels of each figure in (A) and (B) show, for representative cell lines, the cell surface expression of ICAM-1 (solid line) stained with mAb P3.58BA-14 compared to the isotype control antibody upc10 (dotted line) as fluorescence against cell number. The left side shows the relative luciferase expression. The expression of plasmid pIC-174 was set 100% and the activity of the other constructs was compared to this. Plasmid pXP-2 was used as negative control. The standard deviation of at least three independent experiments (see also Table 1) is indicated. (C) Northern blot. 20 µg total RNA of representative cell lines was electrophoresed, blotted and hybridised with either an ICAM-1-specific 690-bp *Pst*I fragment or an oligonucleotide specific for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The size of the up to three ICAM-1 mRNAs (3.4, 2.6 and 1.7 kb) are indicated.

a few cell types [3, 13–16]. However, strong ICAM-1 expression is found on some solid tumours [21–23]. As reported by Simmons et al. [1] and confirmed here, these different ICAM-1 protein levels reflect differences in ICAM-1 mRNA, indicating that this molecule is primarily regulated at the level of transcription. Transient transfection of reporter-gene constructs revealed four regions that appear to be important and indicated that constitutive expression is regulated in two different ways.

The highest levels of reporter-gene expression were observed with the construct pIC-5800. Deletion of the region between positions −5800 and −1352 led to at least a 50% reduction in luciferase activity in all examined cell lines. Using additional constructs, the region mediating this enhancing effect was localised between positions −2400 and −1352, a region previously shown to contain a DNAseI hypersensitive region around position −1550. This site was reported in two ICAM-1-

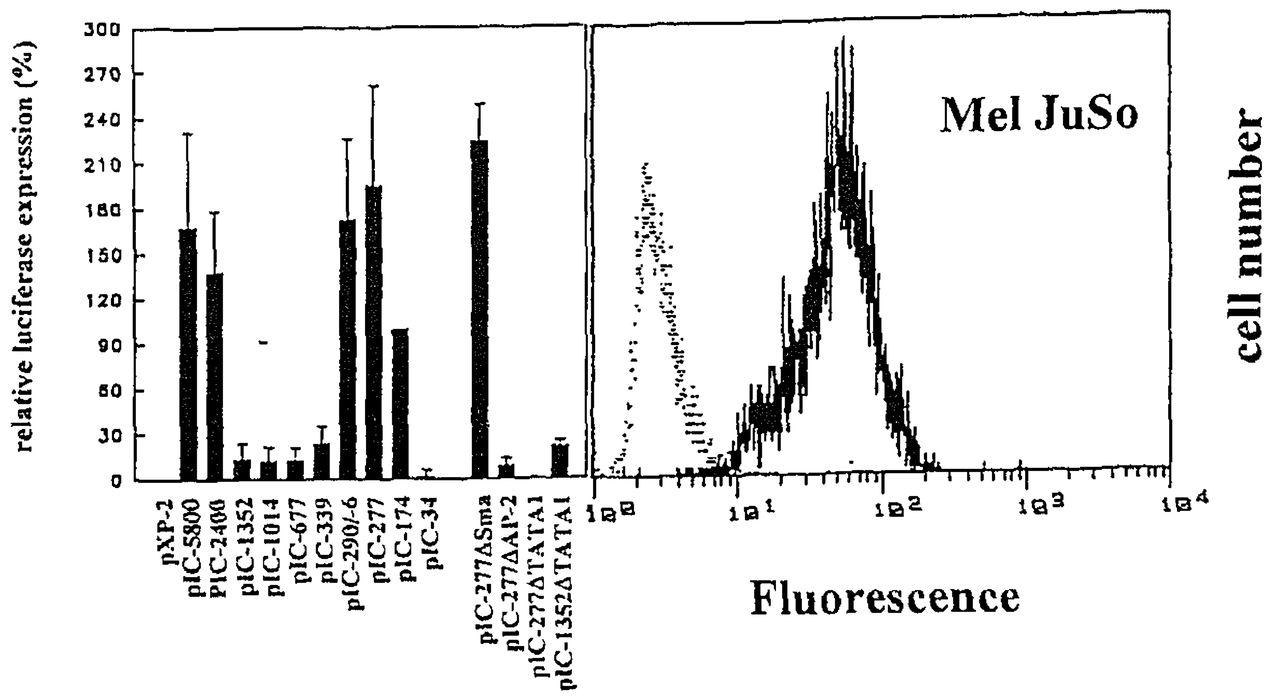

Fig. 3. Location of enhancing and silencing elements in Mel JuSo. Right panel, ICAM-1 expression, as indicated by the antibody staining with anti-(ICAM-1) (solid line, isotype control shown by dotted line). Left panel, relative luciferase activity of the reporter-gene constructs shown on the *x*-axis.

Table 2. Comparison of the ICAM-1 silencing region with published silencer-binding sequences. The silencing region of the human ICAM-1 promoter (positions −339 to −290) shows striking homologies between positions −321 and −302 to different published silencer-binding regions. Bold letters indicate nucleotides identical to the ICAM-1 sequence. The arrows indicate an imperfect inverted repeat. Complementary, complementary to the published sequence.

Sequence	Source	Reference
5′-**ATTTAAAAGTACTTAATAAAC**-3′	ICAM-1 promoter (positions −321 to −302)	37
TATGAAAAGTAGTTATGAAAA	HPV-18 promoter, complementary	51
AAAATTA..TATTAAA	human multiple histocompatability complex class I	47
CTTCATAAC	human α-fetoprotein	52
CAAAAGATAGA	mouse albumin gene, complementary	53
CAAAATACAA**TTA**	mouse N-ras, complementary	54
AAATAAA	rat prolactin gene (position −322)	55
TATAAAATTATA	rat prolactin gene, complementary (position −290)	55

positive cell lines [32], but is also detectable in the ICAM-1-negative cell line LoVo (unpublished observations). An examination of the ICAM-1 sequence between positions −1816 and −1352 for potential transcription-factor-binding sites revealed, at position −1469 (GGGCAAGAGCT), homology to the reverse sequence of an antisilencer-binding site identified in the chicken vimentin gene (TGCCAAGAGCA) [42] as well as homology at position −1783 (CCACCACACC) to an enhancer motif observed in the rat *neu* gene (GGTGGGGGGG) [43]. Two potential NF-κB-binding sites at position −1743 (GGGGTCTCCC) and position −1397 (GGGGCGTCCC), two possible Sp1 sites [positions −1452 (CCGCCC) and −1352 (GGCGGG)] and one putative AP-2 site at position −1415 (CCCAGGCC) were also identified in this region [41].

Further deletion from −1352 to −339 bp led to little or no change in reporter-gene activity in the cells examined. In the so called type-B cells, deletion of the region between positions −339 and −174 also did not alter the luciferase activity. In contrast, in the type-A cells, deletion of this region led to an increase in luciferase expression up to fourfold, indicating the presence of an active silencer element. The silencing influence of this region has been shown previously [29−31] in different cells. On the basis of studies on one ICAM-1-positive cell line that lacked silencer activity and one ICAM-1-negative cell that demonstrated activity, Cornelius et al. [30] concluded that the region from positions −1163 to −277 correlates with the ICAM-1 phenotype and is necessary and sufficient to mediate constitutive ICAM-1 expression. The fact that we found an enhancer upstream of this silencer and the analysis of a large panel of positive and negative cells presented here clearly indicates that the two patterns of reporter-gene activity observed for the region between −339 and −174 bp do not correlate either with the cell type or with the absence or presence of ICAM-1 mRNA or protein. The analysis of additional constructs in Mel JuSo allowed the silencer region to be localised between positions −339 and −290. Computer-aided analysis of this region revealed, around position −313, similarities to silencers found in a variety of different promoters (Table 2), supporting the importance of this region. Although type-B cells generally expressed lower absolute levels of ICAM-1 promoter activity than type-A cells, a B-type pattern was also observed in cells with high absolute activity (e.g. HepG2) and the type-A pattern was observed in cells with low promoter activity (e.g. A-375, H4 and HMCB), indicating that the pattern is not dependent on the absolute reporter-gene activity.

Additional studies carried out in Mel JuSo (Fig. 3) revealed two further regions important in the regulation of the human ICAM-1 gene. The region between positions −277 and −227 (Fig. 3) was found to enhance promoter activity by a factor of two in comparison to pIC-174. A search for transcription-factor-binding motifs in this region revealed the presence of a potential binding site for AP-2 and for NF-κB [41]. Deletion of the DNA from positions −108 to −34, which contains consensus motifs for Sp1 and AP-2, led to a loss of greater than 90% of the

promoter activity in Mel JuSo and, as previously described [28], in cell line 293, suggesting an important role of Sp1 or AP-2 in regulating constitutive ICAM-1 expression. *In-vitro* binding assays did show specific binding of Sp1 to its putative site in all examined cell lines (unpublished observations), underscoring the importance of this region.

ICAM-1 contains two TATA boxes [29]. In Mel JuSo cells, both TATA boxes apparently can be used for the initiation of ICAM-1 transcription. Deletion of the upstream TATA box (Fig. 3, pIC-339 compared to pIC-174) did not decrease the expression of luciferase reporter-gene constructs (in as far as this was detectable because of the overlapping silencer), indicating that transcription is mainly initiated from the downstream start site under constitutive conditions. However, deletion of the downstream TATA box (TATA1) from pIC-1352, a construct containing both TATA boxes, did not alter promoter activity (pIC-1352 compared to pIC-1352ΔTATA1), indicating that, in Mel JuSo, the function of the downstream start site can be replaced by the upstream start site, and suggesting that the silencer functions not only via the downstream TATA box.

Although the presence or absence of an active silencer in the region from positions −339 to −290 could be used to divide the cells examined into two groups, this was not associated with ICAM-1 mRNA or cell surface protein expression. Nevertheless, a relationship between ICAM-1 expression and silencer activity was observed. Silencer activity was detectable only in cells that were ICAM-1 negative or that constitutively expressed high levels of ICAM-1 (type-A cells). All of the cells that showed no silencer activity (type-B cells) expressed low but detectable levels of ICAM-1. These results suggest that at least two distinct mechanisms are involved in the regulation of constitutive ICAM-1 expression. One mechanism, that appears to be associated with inactivity of the silencer region, leads to a low level of constitutive expression such as has been observed in a variety of cells *in vivo* and *in vitro* [3, 13−16] and that may allow ICAM-1 to be rapidly upregulated by cytokines. The second is responsible for the high constitutive levels of ICAM-1, such as is found in macrophages and in some human tumours [21, 23, 24]. This regulation pattern is associated with the presence of an active silencer between positions −339 and −290 that is overridden by the enhancer binding between positions −2400 and −1352.

The transfected full-length 15.5-kb clone HWB 3R1, containing all exons and introns, could be expressed in several ICAM-1-negative cell lines, suggesting that transcription factors acting on regions outside of HWB 3R1 are responsible for constitutive expression, as shown for other genes [44, 45]. This could explain why we failed to identify the region conferring constitutive ICAM-1 expression. Clearly, interactions not imitated by our *in vitro* system may also be involved. Jaiswal et al. [46] detected that limited amounts of tissue-specific factors can be easier titrated out in transient than in stable transfectants. An influence of balanced transcription factors were found in both multiple histocompatability complex class I, where a balance of positive and negative transcription factors seems to regulate its expression [47], as well as in class II, where inhibition of transcription is dependent on the amount of inhibitor Dr1 [48]. Preliminary results (unpublished observations) suggest that DNA methylation, as observed in other genes [49, 50], could participate in regulating constitutive ICAM-1 expression.

The studies reported here reveal important elements influencing ICAM-1 expression and indicate that ICAM-1 is regulated by a complex interaction between positive and negative elements. They also suggest that additional elements outside the examined region and features such as the methylation pattern play an important role in determining the constitutive expression of ICAM-1.

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REFERENCES

1. Simmons, D., Makgoba, M. W. & Seed, B. (1988) ICAM, an adhesion ligand of LFA homologous to the neural cell adhesion molecule NCAM, *Nature 331*, 624−627.
2. Staunton, D. E., Marlin, S. D., Stratowa, C., Dustin, M. L. & Springer, T. A. (1988) primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families, *Cell 52*, 925−933.
3. Rothlein, R., Dustin, M. L., Marlin, S. D. & Springer, T. A. (1986) A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1, *J. Immunol. 137*, 1270−1274.
4. Marlin, S. D. & Springer, T. A. (1987) Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1), *Cell 51*, 813−819.
5. Makgoba, M. W., Sanders, M. E., Ginther-Luce, G. E., Dustin, M. L., Springer, T. A., Clark, E. A., Mannoni, P. & Shaw, S. (1988) ICAM-1 is a ligand for LFA-1 dependent adhesion of B, T, and myeloid cells, *Nature 331*, 86−88.
6. Diamond, M. S., Staunton, D. E., de Fougerolles, A. R., Stacker, S. A., Garcia-Aguillar, J., Hibbs, M. L. & Springer, T. A. (1990) ICAM (CD54) − a counter receptor for Mac-1 (CD11b/CD18), *J. Cell Biol. 111*, 3129−3139.
7. Berendt, A. R., Simmons, D. L., Tansey, J., Newbold, C. I. & Marsh, K. (1989) Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for Plasmodium falciparum, *Nature 341*, 57−59.
8. Staunton, D. E., Merluzzi, V. J., Rothlein, R., Barton, R., Marlin, S. D. & Springer, T. A. (1989) A cell adhesion molecule, ICAM-1 is the major surface receptor for rhinovirus, *Cell 56*, 849−853.
9. Greve, J. M., Meyer, A. M., Forte, C. P., Yost, S. C., Marlor, C. W., Karmack, M. E. & McClelland, A. (1989) The major human rhinovirus receptor is ICAM-1, *Cell 56*, 839−847.
10. Dustin, M. L., Singer, K. H., Tuck, D. T. & Springer, T. A. (1988) Adhesion of T lymphoblasts to epidermal keratinocytes is regulated by interferon γ and is mediated by intercellular adhesion molecule 1 (ICAM-1), *J. Exp. Med. 167*, 1323−1340.
11. Dustin, M. L. & Springer, T. A. (1991) Role of lymphocyte adhesion receptors in transient interactions and cell locomotion, *Annu. Rev. Immunol. 9*, 27−66.
12. Springer, T. A. (1990) Adhesion receptors of the immune system, *Nature 346*, 425−434.
13. Dustin, M. L., Rothlein, R., Bhan, A. K., Dinarello, C. A. & Springer, T. A. (1986) Induction by IL 1 and interferon-γ: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1), *J. Immunol. 137*, 245−254.
14. Rothlein, R., Czajkowski, M., O'Neill, M. M., Marlin, S. D., Mainolfi, E. & Merluzzi, V. J. (1988) Induction of intercellular adhesion molecule 1 on primary and continuous cell lines by proinflammatory cytokines. Regulation by pharmacologic agents and neutralizing antibodies, *J. Immunol. 141*, 1665−1669.
15. Myers, C. L., Wertheimer, S. J., Schembri King, J., Parks, T. & Wallace, R. W. (1992) Induction of ICAM-1 by TNF-alpha, IL-1 beta, and LPS in human endothelial cells after downregulation of PKC, *Am. J. Physiol. 263*, C767−C772.
16. Brockmeyer, C., Ulbrecht, M., Schendel, D. J., Weiss, E. H., Hillebrand, G., Bruckhardt, K., Land, W., Gokel, M. J., Riethmüller, G. & Feucht, H. E. (1993) Distribution of cell adhesion molecules (ICAM-1, VCAM-1, ELAM-1) in renal tissue during allograft rejection, *Transplantation 55*, 610−615.
17. Wegner, C. D., Gundel, R. H., Reilly, P., Haynes, N., Letts, L. G. & Rothlein, R. (1990) Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma, *Science 247*, 456−459.
18. Jevnikar, A. M., Wuthrich, R. P., Takei, F., Xu, H., Brennan, D. C., Glimcher, L. H. & Rubin-Kelley, V. E. (1990) Differing regulation and function of ICAM-1 and class II antigens on renal tubular cells, *Kidney Int. 38*, 417−425.

19. Singer, K. H., Tuck, D. T., Sampson, H. A. & Hall, R. P. (1989) Epidermal keratinocytes express the adhesion molecule intercellular adhesion molecule-1 in inflammatory dermatoses, *J. Invest. Dermatol. 92*, 746–750.
20. Hale, L. P., Martin, M. E., McCollum, D. E., Nunley, J. A., Springer, T. A., Singer, K. H. & Haynes, B. F. (1989) Immunohistologic analysis of the distribution of cell adhesion molecules within the inflammatory synovial microenvironment, *Arth. Rheumatism 32*, 22–29.
21. Johnson, J. P., Stade, B. G., Holzmann, B., Schwäble, W. & Riethmüller, G. (1989) De novo expression of intercellular-adhesion molecule 1 in melanoma correlates with increased risk of metastasis, *Proc. Natl Acad. Sci. USA 86*, 641–644.
22. Johnson, J. P. (1991) The role of ICAM-1 in tumor development, *Chem. Immunol. 50*, 143–63.
23. Natali, P., Nicotra, M. R., Cavaliere, R., Bigotti, A., Romano, G., Temponi, M. & Ferrone, S. (1990) Differential expression of intercellular adhesion molecule 1 in primary and metastatic melanoma lesions, *Cancer Res. 50*, 1271–1278.
24. Vogetseder, W., Feichtinger, T. F., Schulz, W., Schwaeble, W., Tabaczewski, P., Mitterer, M., Böck, G., Marth, C., Dapunt, O., Mikuz, G. & Dierich, M. P. (1989) Expression of 7F7-antigen, a human adhesion molecule identical to intercellular adhesion molecule-1 (ICAM-1) in human carcinomas and their stromal fibroblasts, *Int. J. Cancer 43*, 768–773.
25. Vànky, F., Wang, P., Patarroyo, M. & Klein, E. (1990) Expression of the adhesion molecule ICAM-1 and major histocompatibility complex class I antigens on human tumor cells is required for their interaction with autologous lymphocytes *in vitro*, *Cancer Immunol. Immunother. 31*, 19–27.
26. Webb, D. S., Mostowski, H. S. & Gerrard, T. L. (1991) Cytokine-induced enhancement of ICAM-1 expression results in increased vulnerability of tumor cells to monocyte-mediated lysism, *J. Immunol. 146*, 3682–3686.
27. Pandolfi, F., Trentin, L., Boyle, L. A., Stamenkovic, I., Byers, H. R., Colvin, R. B. & Kurnick, J. T. (1992) Expression of cell adhesion molecules in human melanoma cell lines and their role in cytotoxicity mediated by tumor-infiltrating lymphocytes, *Cancer 69*, 1165–1173.
28. Van de Stolpe, A., Caldenhoven, E., Stade, B. G., Koenderman, L., Raaijmaker, J. A. M., Johnson, J. P. & van der Saag, P. T. (1994) 12-*O*-Tetradecanoylphorbol-13-acetate- and tumor necrosis factor alpha-mediated induction of intercellular adhesion molecule-1 is inhibited by dexamethasone-functional analysis of the human intercellular adhesion molecule-1 promoter, *J. Biol. Chem. 269*, 6185–6192.
29. Voraberger, G., Schafer, R. & Stratowa, C. (1991) Cloning of the human gene for intercellular adhesion molecule 1 and analysis of its 5′-regulatory region. Induction by cytokines and phorbol ester, *J. Immunol. 147*, 2777–2786.
30. Cornelius, L. A., Taylor, J. T., Degitz, K., Li, L.-J., Lawley, T. J. & Caughman, S. W. (1993) A 5′ portion of the ICAM-1 gene confers tissue-specific differential expression levels and cytokine responsiveness, *J. Invest. Dermatol. 100*, 753–758.
31. Degitz, K., Li, L.-J. & Caughman, S. W. (1991) Cloning and characterization of the 5′-transcriptional regulatory region of the human intercellular adhesion molecule 1 gene, *J. Biol. Chem. 266*, 14024–14030.
32. Wawryk, S. O., Cockerill, P. N., Wicks, I. P. & Boyd, A. W. (1991) Isolation and characterization of the promoter region of the human intercellular adhesion molecule-1 gene, *Int. Immunol. 3*, 83–93.
33. Johnson, J. P., Stade, B. G., Hupke, U., Holzmann, B. & Riethmüller, G. (1988) The melanoma progression-associated antigen P3.58 is identical to the intercellular adhesion molecule, ICAM-1, *Immunobiol. 178*, 275–284.
34. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular cloning, a laboratory handbook*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
35. Feinberg, A. P. & Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity, *Anal. Biochem. 132*, 6–13.
36. Dugaiczyk, A., Haron, J. A., Stone, E. M., Dennison, O. E., Rothblum, K. N. & Schwartz, R. J. (1983) Cloning and sequencing of a deoxyribonuclei acid copy of glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid isolated from chicken muscle, *Biochemistry 22*, 1605–1613.
37. Stade, B. G., Messer, G., Riethmüller, G. & Johnson, J. P. (1990) Structural characteristics of the 5′ region of the human ICAM-1 gene, *Immunobiol. 182*, 79–87.
38. Nordeen, S. K. (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers, *Biotechniques 6*, 454–457.
39. Cotten, M., Wagner, E. & Birnstiel, M. L. (1993) Receptor-mediated transport of DNA into eukaryotic cells, *Methods Enzymol. 217*, pp. 45–69.
40. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248–254.
41. Faisst, S. & Meyer, S. (1992) Compilation of vertebrate-encoded transcription factors, *Nucl. Acids Res. 20*, 3–26.
42. Stover, D. M. & Zehner, Z. E. (1992) Identification of a *cis*-acting DNA antisilencer element which modulates vimentin gene expression, *Mol. Cell. Biol. 12*, 2230–2240.
43. Zhao, X. Y. & Hung, M. C. (1992) Negative autoregulation of the *neu* gene is mediated by a novel enhancer, *Mol. Cell. Biol. 12*, 2739–2748.
44. Minie, M., Clark, D., Trainor, C., Evans, T., Reitman, M., Hannon, R., Gould, H. & Felsenfeld, G. (1992) Developmental regulation of globin gene expression, *J. Cell Sci. 16*, 15–20.
45. Dirks, R. P., Jansen, H. J., Onnekink, C., De Jonge, R. J. & Bloemers, H. P. (1993) DNase-I-hypersensitive sites located far upstream of the human *c-sis*/PDGF-B gene comap with transcriptional enhancers and a silencer and are preceded by (part of) a new transcription unit, *Eur. J. Biochem. 216*, 487–95.
46. Jaiswal, A. K., Neuhold, L. A. & Nebert, D. W. (1987) Human P450IA1 upstream regulatory sequences expressing the chloramphenicol acetyltransferase gene. Effect of Ha-MSV enhancer and comparison of transient with stable transformation assays, *Biochem. Biophys. Res. Commun. 148*, 857–863.
47. Weissman, J. D. & Singer, D. S. (1991) A complex regulatory DNA element associated with a major histocompatibility complex class I gene consists of both a silencer and an enhancer, *Mol. Cell. Biol. 11*, 4217–4227.
48. Inostroza, J. A., Mermelstein, F. H., Ha, I., Lane, W. S. & Reinberg, D. (1992) Dr1, a TATA-binding protein-associated phosphoprotein and inhibitor of class II gene transcription, *Cell 70*, 477–489.
49. Shin, T. H., Paterson, A. J., Grant III, J. H., Meluch, A. A. & Kudlow, J. E. (1992) 5-Azacytidine treatment of HA-A melanoma cells induces Sp1 activity and concomitant transforming growth factor alpha expression, *Mol. Cell. Biol. 12*, 3998–4006.
50. Lichtenstein, M., Keini, G., Cedar, H. & Bergman, Y. (1994) B cell-specific demethylation – a novel role for the intronic kappa chain enhancer sequence, *Cell 76*, 913–923.
51. Bauknecht, T., Angel, P., Royer, H.-D. & zur Hausen, H. (1992) Identification of a negative regulatory domain in the human papillomavirus type 18 promoter: interaction with the transcriptional repressor YY1, *EMBO J. 11*, 4607–4617.
52. Nakabayashi, H., Hashimoto, T., Miyao, Y., Tjong, K.-K., Chang, J. & Tamaoki, T. (1991) A position-dependent silencer plays a major role in repressing α-fetoprotein expression in human hepatoma, *Mol. Cell. Biol. 11*, 5885–5893.
53. Herbst, R. S., Boczko, E. M., Darnell, J. E. Jr. & Babiss, L. (1990) The mouse albumin enhancer contains a negative regulatory element that interacts with a novel DNA-binding protein, *Mol. Cell. Biol. 10*, 3896–3905.
54. Paciucci, R., & Pellier, A. (1991) Dissection of the mouse *N-ras* gene upstream regulatory sequences and identification of the promoter and a negative regulatory element, *Mol. Cell. Biol. 11*, 1334–1343.
55. Zhang, Z.-H., Kumar, V., Rivera, R. T., Chisholm, J. & Biswas, D. K. (1990) *cis*-acting negative regulatory element of prolactin gene, *J. Biol. Chem. 265*, 4785–4788.